AMENDMENTS TO THE SPECIFICATION

Please amend each of the below indicated paragraphs and/or sections as shown.

Page 5, lines 17-20

G1

Figure 4A depicts the original overlay of a series of ESTs with some nucleic acid sequence homology to ESTs encoding human placental bikunin, or portions thereof. Shown for reference are the relative positions of bikunin (7-64) (SEQ ID NO: 4) and bikunin (102-159) (SEQ ID NO: 6), labeled KID1 and KID2 respectively.

Page 6, lines 25 to page 7, line 8:

Figure 8 shows the amount of trypsin inhibitory activity present in the cell-free fermentation broth from the growth of yeast strains SC101 (panel 8A) or WHL341 (panel 8B) that were stably transformed with a plasmid (pS604) that directs the expression of placental bikunin (102-159) (SEQ ID NO: 6).

Figure 9 shows both a silver stained SDS-PAGE (left panel) and a Western blot with anti-placental bikunin (102-159) (SEQ ID NO: 6) pAb (right panel) of cell-free fermentation broth from the growth of yeast strain SC101 (recombinants 2.4 and 2.5) that was stably transformed with a plasmid directing the expression of either bovine aprotinin, or placental bikunin (102-159) (SEQ ID NO: 6). Migration was from top to bottom.

G2

Figure 10 is a photograph which shows a silver stained SDS-PAGE of highly purified placental bikunin (102-159) (SEQ ID NO: 6) (lane 2) and a series of molecular size marker proteins (lane 1) of the indicated sizes in Kilodaltons. Migration was from top to bottom.

Figure 11 is a photograph which shows the results of Northern blots of mRNA from various human tissues that was hybridized to a ^{32}P labeled cDNA probe encoding either placental bikunin (102-159) (SEQ ID NO: 6) (panel 11A) or encoding placental bikunin (1-213) (panel 11B). Migration was from top to bottom. The numbers to the right of each blot refer to the size in kilobases of the adjacent RNA markers. The organs from which mRNA was derived is described under each lane of the blot.

Page 7, lines 9-17

Figure 12 depicts an immunoblot of placental derived placental bikunin with rabbit antiserum raised against either synthetic reduced placental bikunin (7-64) (SEQ ID NO: 4) (panel A) or 102-159 (panel B). For each panel, contents were: molecular size markers (lanes 1); native placental bikunin isolated from human placenta (lanes 2); synthetic placental bikunin (7-64) (SEQ ID NO: 4) (lanes 3) and synthetic placental bikunin (102-159) (SEQ ID NO: 6) (lanes 4). Tricine 10-20% SDS-PAGE gels were blotted and developed with protein A-purified primary polyclonal antibody (8 ug IgG in 20 ml 0.1% BSA/Tris-buffered saline (pH 7.5), followed by alkaline phosphatase-conjugated goat anti-rabbit secondary antibody. Migration was from top to bottom.

Page 7, lines 18-21:

Figure 13 depicts a Coomassie Blue stained 10-20% Tricine SDS-PAGE gel of 3 micrograms of highly purified placental bikunin (1-170) (SEQ ID NO: 150) derived from a baculovirus / Sf9 expression system (lane 2). Lane 1 contains molecular size markers. Migration was from top to bottom.

Page 7, lines 22-27:

Figure 14 depicts a comparison of the effect of increasing concentrations of either Sf9-derived human placental bikunin (1-170) (SEQ ID NO: 150) (filled circles), synthetic placental bikunin (102-159) (SEQ ID NO: 6) (open circles), or aprotinin (open squares) on the activated partial thromboplastin time of human plasma. Clotting was initiated with $CaCl_2$. The concentration of proteins are plotted versus the -fold prolongation in clotting time. The uninhibited clotting time was 30.8 seconds.

Page 9, lines 8-19:

A significant advantage of the Kunitz domains of the serine protease inhibitor Bikunin and fragments and analogs thereof of the present invention is that they are human proteins, and also less positively charged than ~~Trasylol®~~ TRASYLOL® (Example 1), thereby reducing the risk of kidney damage on administration of large doses of the proteins. Being of human origin, the protein of the instant invention can thus be administered to human patients with significantly

reduced risk of undesired immunological reactions as compared to administration of similar doses of ~~Trasylol®~~ TRASYLOL®. Furthermore, it was found that bikunin(102-159) (SEQ ID NO: 6), bikunin(7-64) (SEQ ID NO: 4), and bikunin(1-170) (SEQ ID NO: 150) are significantly more potent inhibitors of plasma kallikrein than ~~Trasylol®~~ TRASYLOL® *in vitro* (Example 3, 4 and 10). Thus bikunin and fragments thereof are expected to be more effective *in vivo* relative to aprotinin.

Page 9, line 29 to page 10, line 2:

For stimulating the rates of mucociliary clearance in patients with chronic obstructive lung disease, the proteins of the instant invention may be used like aprotinin ~~Trasylol®~~ TRASYLOL® while taking into account the differences in potency. The use of ~~Trasylol®~~ TRASYLOL® is outlined in the Physicians Desk Reference, 1995, listing for ~~Trasylol®~~ TRASYLOL® supplement A. Briefly, with the patient in a supine position, the loading dose of placental bikunin, isolated domain or other variant is given by infusion slowly over about 20 to 30 minutes. In general, a total dose of between about $2x10^6$ KIU (kallikrein inhibitory units) and 8 $X10^6$ KIU will be used, depending on such factors as patient weight and condition. Preferred loading doses are those that contain a total of 1 to 2 million kallikrein inhibitory units (KIU).

Page 14, line 35 to page 15, line 21:

The existence of a distinct human protein homologous in function to aprotinin, was deduced following a unique analysis of sequence entries to the expressed-sequence-tag data-base (hereafter termed dbEST) at the NCBI (National Center for Biological Information, Maryland). Using the TBlastN algorithm (BLAST, or Basic Local Alignment Search Tool uses the method of Altschul et a., (1990) J. Mol Biol 215, 00 403-410, to search for similarities between a query sequence and all the sequences in a data-base, protein or nucleic acid in any combination), the data-base was examined for nucleotide sequences bearing homology to the sequence of bovine pre-pro-aprotinin, ~~Trasylol®~~ TRASYLOL®. This search of numerous clones was selectively narrowed to two particular clones which could possibly encode for a deduced amino acid sequence that would correspond to a human protein homologous in function to aprotinin. The

selected nucleic acid sequences were R35464 (SEQ ID NO: 12) and R74593 (SEQ ID NO: 14) that were generated from a human placental nucleic acid library. The translated protein sequence in the longest open reading frame for R35464 (SEQ ID NO: 13) was missing one of the 6 cysteines that are critical for formation of the Kunitz-domain covalent structure, meaning that the nucleic acid sequence of R35464 could not yield a functional inhibitor. Similarly, the longest translated open reading frame from clone R74593 (SEQ ID NO: 15) contained a stop codon 5' to the region encoding the Kunitz like sequence, meaning that this sequence, could not be translated to yield a functional secreted Kunitz domain. The significance of these sequences alone was unclear. It was possible that they represented a) the products of pseudogenes, b) regions of untranslated mRNA, or c) the products of viable mRNA which had been sequenced incorrectly.

G7

Page 16, lines 6-17:

Subsequent query of the dbEST for sequences homologous to the Kunitz-like peptide sequence of R74593 yielded H94519 derived from human retina library and N39798. These sequences contained a Kunitz-like sequence that was almost identical to the Kunitz-like domain encoded in R35464 except that it contained all six of the characteristic cysteines. Overlay of each of the nucleotide sequences with that of R74593 (corrected by the insertion of G at b,p, 114) and R35464 was used to obtain a consensus nucleotide sequence for a partial human placental bikunin (SEQ ID NO.: 9; Figure 3). The translated consensus sequence yielded an open reading frame extending from residue -18 to +179 (Figure 3; full translation SEQ ID NO.: 10) that contained two complete Kunitz-like domain sequences, within the region of amino acid residues 17-64 and 102-159 respectively.

G8

Page 18, lines 4-8:

Sequencing of the 872 b.p. fragment showed it to contain nucleotide segment corresponding to b.p. 110 to 218 of EST R87894 at its 5' end and b.p. 310 to 542 of the consensus sequence for placental bikunin inferred from the EST overlay analysis (of Figure 3), at its 3' end. This 3' nucleotide sequence contained all of the Kunitz-like domain encoded by placental bikunin (102-159) (SEQ ID NO: 6).

G9

Page 18, line 37, to page 19, line 24:

To obtain a full length placental bikunin cDNA, the PCR derived product (Figure 4E) was gel purified and used to isolate a non-PCR based full length clone representing the bikunin sequence. The PCR derived cDNA sequence was labeled with ^{32}P-CTP by High Prime (Boehringer Mannheim) and used to probe a placental cDNA Library (Stratagene, Unizap™ λ library) using colony hybridization techniques. Approximately 2 X 10^6 phage plaques underwent 3 rounds of screening and plaque purification. Two clones were deemed full length (~1.5 kilobases) as determined by restriction enzyme analysis and based on comparison with the size of the EST consensus sequence (see above). Sequencing of one of these clone by the dideoxy method yielded the oligonucleotide sequence depicted in Figure 4F. The translation product from this sequence yielded a protein with inframe initiator methionine, signal peptide and mature placental bikunin sequence. The mature placental bikunin sequence was identical to the sequence of the mature protein derived by translation of the EST consensus although the signal peptide sequence lengths and sequences differed. Unlike the PCR derived product, the cDNA derived by colony hybridization contained the entire ectodomain, transmembrane domain, cytoplasmic domain and in-frame stop codon. In fact, the clone extended all the way to the poly-A tail. The initiator methionine was followed by a hydrophobic signal peptide which was identical to the signal peptide encoded in the PCR derived clone. Subsequently we expressed and purified a soluble fragment of placental bikunin, bikunin (1-170) (SEQ ID NO: 150), from Sf9 cells (Example 9), and found it to be a functional protease inhibitor (Example 10). Furthermore, we isolated from human placenta a soluble fragment of placental bikunin which was also an active protease inhibitor (Example 7).

G10

Page 20, lines 2-15:

The amino acid sequences for placental bikunin (7-64) (SEQ ID NO: 4), bikunin (102-159) (SEQ ID NO: 6), and full length placental bikunin (Figure 4F) were searched against the PIR (Vers. 46.0) and PatchX (Vers. 46.0) protein databases as well as the GeneSeq (Vers. 20.0) protein database of patented sequences using the Genetics Computer Group program FastA. Using the Genetics Computer Group program TFastA (Pearson and Lipman, 1988, Proc. Natl. Acad. Sci. USA 85:2444-2448), these same protein sequences were searched versus the six-

G11

frame translations of the GenBank (Vers. 92.0 with updates to 1/26/96) and EMBL (modified Vers. 45.0) nucleotide databases as well as the GeneSeq (Vers. 20.0) nucleotide database of patented sequences. The EST and STS subsets of GenBank and EMBL were not included in this set of searches. The best matches resulting from these searches contained sequences which were only about 50% identical over their full length to the 58-amino acid protein sequence derived from our analysis of clones R74593 and R35464.

G11

Page 20, lines 18-23:

As mentioned above, synthetic peptides corresponding to bikunin (7-64) (SEQ ID NO: 4) and bikunin (102-159) (SEQ ID NO: 6) as determined from the translated consensus sequence for bikunin (Figure 3), could be refolded (Examples 2 and 1, respectively) to yield active kallikrein inhibitor protein (Example 4 and 3, respectively). We exploited this unexpected property to devise a purification scheme to isolate native placental bikunin from human tissue.

G12

Page 25, lines 16-22:

One can recognize that the individual Kunitz-like domains are also fragments of the native placental bikunin. In particular, the instant invention contemplates the use of a protein having the amino acid sequence of a first Kunitz-like domain consisting of the amino acid sequence of native human placental bikunin amino acids 7-64, hereinafter called "bikunin (7-64) (SEQ ID NO: 4)". Thus in one embodiment the instant invention encompasses a protein which contains at least one Kunitz-like domain having the amino acid sequence:

G13

Page 25, line 42, to page 26, line 2:

The instant invention also provides for a protein having the amino acid sequence of a Kunitz-like domain consisting of the amino acid sequence of native human placental bikunin amino acids 102-159, hereinafter called "bikunin (102-159) (SEQ ID NO: 6)". Thus one embodiment the instant invention encompasses a protein which contains at least one Kunitz-like domain having the amino acid sequence:

G14

Page 29, lines 12-19:

The present invention also provides for a method for stimulating MCC that employs variants of placental bikunin, and the specific Kunitz domains described above, that contain amino acid substitutions that alter the protease specificity. Preferred sites of substitution are indicated below as positions Xaa^{1} through Xaa^{32} in the amino acid sequence for native placental bikunin. Substitutions at Xaa^{1} through Xaa^{16} are also preferred for variants of bikunin (7-64) (SEQ ID NO: 4), while substitutions at Xaa^{17} through Xaa^{32} are preferred for variants of bikunin (102-159) (SEQ ID NO: 6).

G15

Page 30, lines 18-24:

By substituting one or more amino acids in one or more of the positions indicated above, it may be possible to change the inhibitor specificity profile of native placental bikunin or that of the individual Kunitz-like domains, bikunin(7-64) (SEQ ID NO: 4) or bikunin (102-159) (SEQ ID NO: 6) so that it preferentially inhibits other serine proteases such as, but not limited to, the enzymes of the complement cascade, TF/FVIIa, FXa, prostasin, thrombin, neutrophil elastase, cathepsin G or proteinase-3.

G16

Page 32, line 36, to page 33, line 18:

The instant invention also relates to transformed cells containing the DNA constructs encoding the placental bikunin, isolated domains or other variants of the present invention that can be used for the production of recombinant placental bikunin variants. A variety of combinations of expression vector and host organism exist which can be used for the production of the placental bikunin variants. Suitable host cells include baculovirus infected Sf9 insect cells, mammalian cells such as BHK, CHO, Hela and C-127, bacteria such as E. coli, and yeasts such as Saccharomyces cervisiae. Methods for the use of mammalian, insect and microbial expressions systems needed to achieve expression of placental bikunin are well known in the art and are described, for example, in Ausubel F.M et al., Current Protocols in Molecular Biology, John Wiley & Sons (1995), Chapter 16. For fragments of placental bikunin containing a single Kunitz inhibitor domain such as bikunin (7-64) (SEQ ID NO: 4) and (102-159) (SEQ ID NO: 6), yeast and E. coli expression systems are preferable, with yeast systems being most preferred.

G17

Typically, yeast expression would be carried out as described in US patent 5,164,482 for aprotinin variants and adapted in Example 5 of the present specification for placental bikunin (102-159) (SEQ ID NO: 6). E.coli expression could be carried out using the methods described in US patent 5,032,573. Use of mammalian and yeast systems are most preferred for the expression of larger placental bikunin variants containing both inhibitor domains such as the variant bikunin(7-159).

Page 33, line 36, to page 34, line 4:

Preparation of synthetic placental bikunin (102-159) (SEQ ID NO: 6)

Materials and methods/Reagents used. The fluorogenic substrate Tos-Gly-Pro-Lys-AMC was purchased from Bachem BioScience Inc (King of Prussia, PA). PNGB, Pro-Phe-Arg-AMC, Ala-Ala-Pro-Met-AMC (where AMC represents 7-amino-4-methylcoumarin), bovine trypsin (type III), human plasma kallikrein, and human plasmin were from Sigma (St. Louis, MO).

Page 34, lines 5-8:

Recombinant aprotinin (~~Trasylol®~~ TRASYLOL®) was from Bayer AG (Wuppertal, Germany). Pre-loaded Gln Wang resin was from Novabiochem (La Jolla, CA). Thioanisole, ethanedithiol and t-butyl methyl ether was from Aldrich (Milwaukee, WI).

Page 34, lines 10-23:

Quantification of functional placental bikunin (7-64) (SEQ ID NO: 4) and (102-159) (SEQ ID NO: 6)

The amount of trypsin inhibitory activity present in the refolded sample at various stages of purification was measured using GPK-AMC as a substrate. Bovine trypsin (200 pmoles) was incubated for 5 min at 37%C with bikunin (7-64) (SEQ ID NO: 4) or (102-159) (SEQ ID NO: 6), from various stages of purification, in buffer A (50 mM Hepes, pH 7.5, 0.1 M NaCl, 2 mM $CaCl_2$ and 0.01% triton X-100). GPK-AMC was added (20 μM final) and the amount of coumarin produced was determined by measuring the fluorescence (ex = 370 nm, em = 432 nm) on a Perkin-Elmer LS-50B fluorimeter over a 2 min. period. For samples being tested the % inhibition for each was calculated according to equation 1; where R_o is the rate of

G20 fluorescence increase in the presence of inhibitor and R_1 is the rate determined in the absence of added sample. One unit of activity for the inhibitor is defined as the amount needed to achieve 50% inhibition in the assay using the conditions as described.

Page 34, lines 27-37:

Synthesis. Placental bikunin (102-159) (SEQ ID NO: 6) was synthesized on an Applied Biosystems model 420A peptide synthesizer using NMP-HBTU Fmoc chemistry. The peptide was synthesized on pre loaded Gln resin with an 8-fold excess of amino acid for each coupling. Cleavage and deprotection was performed in 84.6% trifluoroacetic acid (TFA), 4.4% thioanisole, G21 2.2% ethanedithiol, 4.4% liquified phenol, and 4.4% H_2O for 2 hours at room temperature. The crude peptide was precipitated, centrifuged and washed twice in t-butyl methyl ether. The peptide was purified on a Dynamax 60A C18 reverse-phase HPLC column using a TFA/acetonitrile gradient. The final preparation (61.0 mg) yielded the correct amino acid composition and molecular mass by Electrospray mass spectroscopy (MH+ =6836.1; calcd = 6835.5) for the predicted sequence:

Page 35, lines 5-29:

Purification. Refolding of placental bikunin (102-159) (SEQ ID NO: 6) was performed according to the method of Tam et al., (J. Am. Chem. Soc. 1991, 113; 6657-62). A portion of the purified peptide (15.2 mg) was dissolved in 4.0 ml of 0.1 M Tris, pH 6.0, and 8 M urea. Oxidation of the disulfides was accomplished by dropwise addition of a solution containing 23% DMSO, and 0.1 M Tris, pH 6.0 to obtain a final concentration of 0.5 mg/ml peptide in 20% G22 DMSO, 0.1 M Tris, pH 6.0, and 1 M urea. The solution was allowed to stir for 24 hr at 25°C after which it was diluted 1:10 in buffer containing 50 mM Tris, pH 8.0, and 0.1 M NaCl. The material was purified using a kallikrein affinity column made by covalently attaching 30 mg of bovine pancreatic kallikrein (Bayer AG) to 3.5 mls of CNBr activated Sepharose (Pharmacia) according to the manufacturers instructions. The refolded material was loaded onto the affinity column at a flow rate of 1 ml/min and washed with 50 mM Tris, pH 8.0, and 0.1 M NaCl until absorbance at 280 nm of the wash could no longer be detected. The column was eluted with 3 volumes each of 0.2 M acetic acid, pH 4.0 and 1.7. Active fractions were pooled (see below)

and the pH of the solution adjusted to 2.5. The material was directly applied to a Vydac C18 reverse-phase column (5 micron, 0.46 x 25 cm) which had been equilibrated in 22.5% acetonitrile in 0.1% TFA. Separation was achieved using a linear gradient of 22.5 to 40% acetonitrile in 0.1% TFA at 1.0 ml/min over 40 min. Active fractions were pooled, lyophilized, redissolved in 0.1% TFA, and stored at -20°C until needed.

G22

Results. Synthetic placental bikunin (102-159) (SEQ ID NO: 6) was refolded using 20% DMSO as the oxidizing agent as described above, and purified by a 2-step purification protocol as shown below, to yield an active trypsin inhibitor (Table 1 below).

Page 36, line 1:

G23

Table 1 Purification table for the isolation of synthetic placental bikunin (102-159) (SEQ ID NO: 6)

Page 36, lines 10-18:

Chromatography of the crude refolded material over an immobilized bovine pancreatic kallikrein column selectively isolated 6.0% of the protein and 97% of the trypsin inhibitory activity present. Subsequent chromatography using C18 reverse-phase yielded a further purification of 2-fold, with an overall recovery of 74%. On RPHPLC, the reduced and refolded placental bikunin (102-159) (SEQ ID NO: 6), exhibited elution times of 26.3 and 20.1 minutes, respectively. Mass spectroscopy analysis of the purified material revealed a molecular mass of 6829.8; a loss of 6 mass units from the starting material. This demonstrates the complete formation of the 3 disulfides predicted from the peptide sequence.

G24

Page 36, lines 19-30:

The isoelectric points of the purified, refolded synthetic placental bikunin (102-159) (SEQ ID NO: 6) was determined using a Multiphor II Electrophoresis System (Pharmacia) run according to the manufacturers suggestions, together with pI standards, using a precast ~~Ampholine®~~ AMPHOLINE® PAGplate (pH 3.5 to 9.5) and focused for 1.5 hrs. After staining, the migration distance from the cathodic edge of the gel to the different protein bands was measured. The pI of each unknown was determined by using a standard curve generated by a plot of the migration distance of standards versus the corresponding pI's. With this technique, the pI of placental bikunin (102-159) (SEQ ID NO: 6) was determined to be 8.3, in agreement with

G25

G25 the value predicted from the amino acid sequence. This is lower than the value of 10.5 established for the pI of aprotinin. (Tenstad et al., 1994, Acta Physiol. Scand. 152:33-50).

Page 37, lines 2-10:

Preparation of synthetic placental bikunin (7-64) (SEQ ID NO: 4)

Placental bikunin (7-64) (SEQ ID NO: 4) was synthesized, refolded and purified essentially as described for placental bikunin (102-159) (SEQ ID NO: 6) but with the following

G26 modifications: during refolding, the synthetic peptide was stirred for 30 hr as a solution in 20% DMSO at 25°C; purification by C18 RP-HPLC was achieved with a linear gradient of 25 to 45% acetonitrile in 0.1% TFA over 40 min (1ml/min). Active fractions from the first C18 run were reapplied to the column and fractionated with a linear gradient (60 min, 1 ml/min) of 20 to 40% acetonitrile in 0.1% TFA.

Page 37, line 21:

G27 **Table 2A Purification table for the isolation of synthetic placental bikunin (7-64) (SEQ ID NO: 4)**

Page 37, line 27 to page 38, line 1:

The pI of placental bikunin (7-64) (SEQ ID NO: 4) was determined using the methods employed to determine the pI of placental bikunin (102-159) (SEQ ID NO: 6). Placental bikunin

G28 (7-64) (SEQ ID NO: 4) exhibited a pI that was much higher than the predicted value (pI = 7.9). Refolded placental bikunin (7-64) (SEQ ID NO: 4) migrated to the cathodic edge of the gel (pH 9.5) and an accurate pI could not be determined under these conditions.

Page 38, lines 3-11:

Continued Preparation of synthetic placental bikunin (7-64) (SEQ ID NO: 4)

Because the synthetic placental bikunin (7-64) (SEQ ID NO: 4) may not have undergone

G29 complete deprotection prior to purification and refolding, refolding was repeated using protein which was certain to be completely deprotected. Placental bikunin (7-64) (SEQ ID NO: 4) was synthesized, refolded and purified essentially as described for placental bikunin (102-159) (SEQ ID NO: 6) but with the following modifications: during refolding, the synthetic peptide (0.27

mg/ml) was stirred for 30 hr as a solution in 20% DMSO at 25 C; purification by C18 RP-HPLC was achieved with a linear gradient of 22.5 to 50% acetonitrile in 0.1% TFA over 40 min (1 ml/min).

Page 38, line 21:

Table 2B Purification table for the isolation of synthetic placental bikunin (7-64) (SEQ ID NO: 4)

Page 38, lines 27-30:

The pI of refolded placental bikunin (7-64) (SEQ ID NO: 4) was determined using the methods employed to determine the pI of placental bikunin (102-159) (SEQ ID NO: 6). Refolded placental bikunin (7-64) (SEQ ID NO: 4) exhibited a pI of 8.85, slightly higher than the predicted value (pI = 7.9).

Page 39, line 2:

In vitro specificity of functional placental bikunin fragment (102-159) (SEQ ID NO: 6)

Page 39,lines 16-37:

Inhibition Kinetics: The inhibition of trypsin by placental bikunin (102-159) (SEQ ID NO: 6) or aprotinin was measured by the incubation of 50 pM trypsin with placental bikunin (102-159) (SEQ ID NO: 6) (0-2 nM) or aprotinin (0-3 nM) in buffer A in a total volume of 1.0 ml. After 5 min. at 37°C, 15 µl of 2 mM GPK-AMC was added and the change in fluorescence (as above) was monitored. The inhibition of human plasmin by placental bikunin (102-159) (SEQ ID NO: 6) and aprotinin was determined with plasmin (50 pM) and placental bikunin (102-159) (SEQ ID NO: 6) (0-10 nM) or aprotinin (0-4 nM) in buffer containing 50 mM Tris-HCl (pH 7.5), 0.1 M NaCl, and 0.02% triton x-100. After 5 min. incubation at 37°C, 25 µl of 20 mM GPK-AMC was added and the change in fluorescence monitored. The inhibition of human plasma kallikrein by placental bikunin (102-159) (SEQ ID NO: 6) or aprotinin was determined using kallikrein (2.5 nM) and placental bikunin (102-159) (SEQ ID NO: 6) (0-3 nM) or aprotinin (0-45 nM) in 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, and 0.02% triton x-100. After 5 min. at

37°C 15 µl of 20 mM PFR-AMC was added and the change in fluorescence monitored. The inhibition of bovine pancreatic kallikrein by placental bikunin (102-159) (SEQ ID NO: 6) and aprotinin was determined in a similar manner with kallikrein (92 pM), placental bikunin (102-159) (SEQ ID NO: 6) (0-1.6 nM) and aprotinin (0-14 pM) and a final substrate concentration of 100 µM. The apparent inhibition constant K_i* was determined using the nonlinear regression data analysis program Enzfitter software (Biosoft, Cambridge, UK): The kinetic data from each experiment were analyzed in terms of the equation for a tight binding inhibitor:

Page 40, lines 12-18:

For the inhibition of human neutrophil elastase by placental bikunin (102-159) (SEQ ID NO: 6) and aprotinin, elastase (19 nM) was incubated with placental bikunin (102-159) (SEQ ID NO: 6) (150 nM) or aprotinin (0-7.5 µM) in buffer containing 0.1 M Tris-HCl (pH 8.0), and 0.05% triton X-100. After 5 min at 37%C, AAPM-AMC (500 µM or 1000 µM) was added and the fluorescence measured over a two-minute period. Ki values were determined from Dixon plots of the form 1/V versus [I] performed at two different substrate concentrations (Dixon et al., 1979).

Page 40, line 19 to page 41, line 10:

The inhibition of human tissue kallikrein by aprotinin, placental bikunin fragment (7-64) (SEQ ID NO: 4) or placental bikunin fragment (102-159) (SEQ ID NO: 6) was measured by the incubation of 0.35 nM human tissue kallikrein with placental bikunin (7-64) (SEQ ID NO: 4) (0-40 nM) or placental bikunin (102-159) (SEQ ID NO: 6) (0-2.5 nM), or aprotinin (0-0.5 nM) in a 1 ml reaction volume containing 50 mM Tris-HCl buffer pH 9.0, 50 mM NaCl, and 0.1% triton x-100. After 5 min. at 37°C, 5 ul of 2 mM PFR-AMC was added achieving 10 uM final and the change in fluorescence monitored. The Km for PFR-AMC with human tissue kallikrein under the conditions employed was 5.7 uM. The inhibition of human factor Xa (American Diagnostica, Inc, Greenwich, CT) by synthetic placental bikunin (102-159) (SEQ ID NO: 6), recombinant placental bikunin, and aprotinin was measured by the incubation of 0.87 nM human factor Xa with increasing amounts of inhibitor in buffer containing 20 mM Tris (pH 7.5), 0.1 M NaCl, and 0.1% BSA. After 5 min. at 37°C, 30 ul of 20 mM LGR-AMC (Sigma) was added and the change

in fluorescence monitored. The inhibition of human urokinase (Sigma) by Kunitz inhibitors was measured by the incubation of urokinase (2.7 ng) with inhibitor in a total volume of 1 ml buffer containing 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, and 0.1% Triton x-100. After 5 min. at 37°C, 35 ul of 20 mM GGR-AMC (Sigma) was added and the change in fluorescence monitored. The inhibition of Factor XIa (from Enzyme Research Labs, Southbend, IN) was measured by incubating FXIa (0.1 nM) with either 0 to 800 nM placental bikunin (7-64) (SEQ ID NO: 4), 0 to 140 nM placental bikunin (102-159) (SEQ ID NO: 6) or 0 to 40 uM aprotinin in buffer containing 50 mM Hepes pH 7.5, 100 mM NaCl, 2 mM CaCl2, 0.01% triton x-100, and 1% BSA in a total volume of 1 ml. After 5 min at 37 C, 10 ul of 40 mM Boc-Glu(OBzl)-Ala-Arg-AMC (Bachem Biosciences, King of Prussia, PA) was added and the change in fluorescence monitored.

Results: A direct comparison of the inhibition profiles of placental bikunin (102-159) (SEQ ID NO: 6) and aprotinin was made by measuring their inhibition constants with various proteases under identical conditions. The K_i values are listed in Table 3 below.

Page 42, lines 1-21:

Table 3 Ki values for the inhibition of various proteases by bikunin (102-159) (SEQ ID NO: 6)

TABLE 3

Protease (concentration)	bikunin (102-159) (SEQ ID NO: 6) Ki (nM)	Aprotinin Ki (nM)	Substrate (concentration)	Km (mM)
Trypsin (48.5 pM)	0.4	0.8	GPK-AMC (0.03 mM)	0.022
Chymotrypsin (5 nM)	0.24	0.86	AAPF-pNA (0.08 mM)	0.027
Bovine Pancreatic Kallikrein (92.0 pM)	0.4	0.02	PFR-AMC (0.1 mM)	0.08
Human Plasma Kallikrein (2.5 nM)	0.3	19.0	PFR-AMC (0.3 mM)	0.46
Human Plasmin (50 pM)	1.8	1.3	GPK-AMC (0.5 mM)	0.73
Human Neutrophil Elastase (19 nM)	323.0	8500.0	AAPM-AMC (1.0 μM)	1.6
Factor XIIa	>300.0	12,000.0	PFR-AMC (0.2 μM)	0.35
Human Tissue Kallikrein (0.35 nM)	0.13	0.004	PFR-AMC (10 μM)	0.0057
factor Xa (0.87 nM)	274	N.I. at 3 μM	LGR-AMC (0.6 mM)	N.D.
urokinase	11000	4500	GGR-AMC (0.7 mM)	N.D.

factor XIa (0.1 nM)	15	288	E(OBz)AR-AMC (0.4 mM)	0.46

Placental bikunin (102-159) (SEQ ID NO: 6) and aprotinin inhibit bovine trypsin and human plasmin to a comparable extent under the conditions employed. Aprotinin inhibited elastase with a Ki of 8.5 μM. Placental bikunin (102-159) (SEQ ID NO: 6) inhibited elastase with a Ki of 323nM. The K_i value for the placental bikunin (102-159) (SEQ ID NO: 6) inhibition of bovine pancreatic kallikrein was 20-fold higher than that of aprotinin inhibition. In contrast, placental bikunin (102-159) (SEQ ID NO: 6) is a more potent inhibitor of human plasma kallikrein than aprotinin and binds with a 56-fold higher affinity.

G36 Cont

Because placental bikunin (102-159) (SEQ ID NO: 6) is greater than 50 times more potent than ~~Trasylol®~~ TRASYLOL® as an inhibitor of kallikrein, smaller amounts of human placental bikunin, or fragments thereof (i.e. placental bikunin (102-159) (SEQ ID NO: 6)) are needed than ~~Trasylol®~~ TRASYLOL® in order to maintain the effective patient doses of inhibitor in KIU. This reduces the cost per dose of the drug and reduces the likelihood of adverse nephrotoxic effects upon re-exposure of the medicament to patients. Furthermore, the protein is human derived, and thus much less immunogenic in man than aprotinin which is derived from cows. This results in significant reductions in the risk of incurring adverse immunologic events upon re-exposure of the medicament to patients.

Page 43, lines 3-6:

In vitro specificity of functional placental bikunin fragment (7-64) (SEQ ID NO: 4)

G37

In vitro specificity of functional human placental bikunin (7-64) (SEQ ID NO: 4) was determined using the materials and methods as described in the Examples above.

Page 43, lines 7-10:

Results: The table below shows the efficacy of placental bikunin (7-64) (SEQ ID NO: 4) as an inhibitor of various serine proteases *in vitro*. Data is shown compared against data obtained for screening inhibition using either placental bikunin (102-159) (SEQ ID NO: 6), or aprotinin (~~Trasylol®~~ TRASYLOL®).

G38

Page 43, line 12 to page 44, line 9:

Table 4 A Ki values for the inhibition of various proteases by bikunin(7-64) (SEQ ID NO: 4)

TABLE 4A			
Protease (concentration)	bikunin(7-64) (SEQ ID NO: 4) Ki (nM)	Aprotinin Ki (nM)	Bikunin (102-159) (SEQ ID NO: 6) Ki (nM)
Trypsin (48.5 pM)	0.17	0.8	0.4
Bovine Pancreatic Kallikrein (92.0 pM)	0.4	0.02	0.4
Human Plasma Kallikrein (2.5 nM)	2.4	19.0	0.3
Human Plasmin (50 pM)	3.1	1.3	1.8
Bovine chymotrypsin (5 nM)	0.6	0.9	0.2
Factor XIIa	>300	12000	>300
elastase	>100	8500	323

The results show that the amino acid sequence encoding placental bikunin (7-64) (SEQ ID NO: 4) can be refolded to obtain an active serine protease inhibitor that is effective against at least four trypsin-like serine proteases.

G39

Table 4B below also shows the efficacy of refolded placental bikunin (7-64) (SEQ ID NO: 4) as an inhibitor of various serine proteases *in vitro*. Refolded placental bikunin (7-64) (SEQ ID NO: 4) was prepared from protein that was certain to be completely deprotected prior to purification and refolding. Data is shown compared against data obtained for screening inhibition using either placental bikunin (102-159) (SEQ ID NO: 6), or aprotinin (~~Trasylol~~® TRASYLOL®).

Table 4B Ki values for the inhibition of various proteases by refolded bikunin (7-64) (SEQ ID NO: 4)

TABLE 4B			
Protease (concentration)	bikunin (7-64) ID NO: 4) Ki (nM)	Aprotinin Ki (nM)	bikunin (102-159) (SEQ ID NO: 6) Ki (nM)
Trypsin (50 pM)	0.2	0.8	0.3
Human Plasma Kallikrein (0.2 nM)	0.7	19.0	0.7
Human Plasmin (50 pM)	3.7	1.3	1.8
Factor XIIa	not done	12,000	4,500
Factor XIa (0.1 nM)	200	288	15
Human Tissue Kallikrein	2.3	0.004	0.13

G39 Suprisingly, placental bikunin (7-64) (SEQ ID NO: 4) was more potent than aprotinin at inhibiting human plasma kallikrein, and at least similar in efficacy as a plasmin inhibitor. These data show that placental bikunin (7-64) (SEQ ID NO: 4) is at least as effective as aprotinin, using *in vitro* assays, and that one would expect better or similar potency *in vivo*.

Page 44, lines 12-23:

Expression of placental bikunin variant (102-159) (SEQ ID NO: 6) in yeast

The DNA sequence encoding placental bikunin 102-159 (SEQ ID NO.: 6) was generated using synthetic oligonucleotides. The final DNA product consisted (5' to 3') of 15 nucleotides G40 from the yeast α-mating factor propeptide sequence fused to the in-frame cDNA sequence encoding placental bikunin (102-159) (SEQ ID NO: 6), followed by an in-frame stop codon. Upon cloning into a yeast expression vector pS604, the cDNA would direct the expression of a fusion protein comprising an N-terminal yeast α-mating factor propeptide fused to the 58 amino acid sequence of placental bikunin (102-159) (SEQ ID NO: 6). Processing of this fusion protein at a KEX-2 cleavage site at the junction between the α-mating factor and Kunitz domain was designed to liberate the Kunitz domain at its native N-terminus.

Page 45, lines 10-33:

The oligonucleotides were dissolved in 10 mM Tris buffer pH 8.0 containing 1 mM EDTA, and 12 ug of each oligo were added combined and brought to 0.25M NaCl. To hybridize, the oligonucleotides were denatured by boiling for 5 minutes and allowed to cool from 65°C to room temp over 2 hrs. Overlaps were extended using the Klenow fragment and digested with HindIII and BamHI. The resulting digested double stranded fragment was cloned into pUC19 G41 and sequence confirmed. A clone containing the fragment of the correct sequence was digested with BamHI/HindIII to liberate the bikunin containing fragment with the following + strand sequence:

GAA GGG GTA AGC TTG GAT AAA AGA TAT GAA GAA TAC TGC ACC GCC AAC
GCA GTC ACT GGG CCT TGC CGT GCA TCC TTC CCA CGC TGG TAC TTT GAC
GTG GAG AGG AAC TCC TGC AAT AAC TTC ATC TAT GGA GGC TGC CGG GGC
AAT AAG AAC AGC TAC CGC TCT GAG GAG GCC TGC ATG CTC CGC TGC TTC
CGC CAG TAG GGA TCC (SEQ ID.: 44)

which was then gel purified and ligated into BamHI/HindIII cut pS604. The ligation mixture was extracted into phenol/chloroform and purified over a S-200 minispin column. The ligation product was directed transformed into yeast strains SC101 and WHL341 and plated on ura selection plates. Twelve colonies from each strain were re-streaked on ura drop out plates. A single colony was inoculated into 2 ml of ura DO media and grown over night at 30°C. Cells were pelleted for 2 minutes at 14000x g and the supernatants evaluated for their content of placental bikunin (102-159) (SEQ ID NO: 6).

G41

Page 45, line 35:

G42

Detection of expression of placental bikunin (102-159) (SEQ ID NO: 6) in transformed yeast

Page 46, line 2 to page 48, line 17:

The second method to quantify placental bikunin (102-159) (SEQ ID NO: 6) expression exploited use of polyclonal antibodies (pAbs) against the synthetic peptide to monitor the accumulation of the recombinant peptide using Western blots. These studies were performed only with recombinants derived from strain SC101, since these produced greater inhibitory activity than recombinants derived from strain WHL341.

To produce the pAb, two 6-8 week old New Zealand White female rabbits (Hazelton Research Labs, Denver, Pa) were immunized on day zero with 250 ug of purified reduced synthetic placental bikunin (102-159) (SEQ ID NO: 6), in Complete Freund's adjuvant, followed by boosts on days 14, 35 and 56 and 77 each with 125 ug of the same antigen in Incomplete Freund's adjuvant. Antiserum used in the present studies was collected after the third boost by established procedures. Polyclonal antibodies were purified from the antiserum over protein A.

G43

Colonies 2.4 and 2.5 from transformation of yeast SC101 (Figure 8) as well as an aprotinin control were grown overnight in 50 ml of ura DO media at 30°C. Cells were pelleted and the supernatant concentrated 100-fold using a Centriprep 3 (Amicon, Beverly, MA) concentrator. Samples of each (30 µl) were subjected to SDS-PAGE on 10-20% tricine buffered gels (Novex, San Diego, CA) using the manufacturers procedures. Duplicate gels were either developed with a silver stain kit (Integrated Separation Systems, Nantick, MA) or transferred to

nitrocellulose and developed with the purified polyclonal antibody elicited to synthetic bikunin (102-159) (SEQ ID NO: 6). Alkaline-phosphatase conjugated goat anti-rabbit antibody was used as the secondary antibody according to the manufacturer's directions (Kirkegaard and Perry, Gaithersburg, MD).

Purification of placental bikunin (102-159) (SEQ ID NO: 6) from a transformed strain of SC101

Fermentation broth from a 1L culture of SC101 strain 2.4 was harvested by centrifugation (4,000 g x 30 min.) then applied to a 1.0 ml column of anhydrochymotrypsin-sepharose (Takara Biochemical Inc., CA), that was previously equilibrated with 50 mM Hepes buffer pH 7.5 containing 0.1M NaCl, 2 mM $CaCl_2$ and 0.01% (v/v) triton X-100. The column was washed with the same buffer but containing 1.0 M NaCl until the A280nm declined to zero, whereupon the column was eluted with 0.1M formic acid pH 2.5. Eluted fractions were pooled and applied to a C18 column (Vydac, 5um, 4.6 x 250 mm) previously equilibrated with 0.1% TFA, and eluted with a 50 min. linear gradient of 20 to 80% acetonitrile in 0.1% TFA. Fractions containing placental bikunin (102-159) (SEQ ID NO: 6) were pooled and re-chromatographed on C18 employing elution with a linear 22.5 to 50% acetonitrile gradient in 0.1% TFA.

Results.Figure 8 shows the percent trypsin activity inhibited by twelve colonies derived from the transformation of each of strains SC101 and WHL341. The results show that all twelve colonies of yeast strain SC101 transformed with the trypsin inhibitor placental bikunin (102-159) (SEQ ID NO: 6) had the ability to produce a substantial amount of trypsin inhibitory activity compared to the negative controls both of which showed no ability to inhibit trypsin. The activity is therefore related to the expression of a specific inhibitor in the placental bikunin variant (102-159) (SEQ ID NO: 6) transformed cells. The yeast WHL341 samples contained minimal trypsin inhibitory activity. This may be correlated to the slow growth observed with this strain under the conditions employed.

G43 Cont

Figure 9 shows the SDS-PAGE and western analysis of the yeast SC101 supernatants. Silver stained SDS-PAGE of supernatants derived from recombinant yeasts 2.4 and 2.5 expressing placental bikunin (102-159) (SEQ ID NO: 6) as well as from the yeast expressing aprotinin yielded a protein band running at approximated 6 kDa, corresponding to the size expected for each recombinant Kunitz inhibitor domain. Western analysis showed that the 6 kDa bands expressed by stains 2.4. and 2.5 reacted with the pAb elicited to placental bikunin (102-159) (SEQ ID NO: 6). The same 6 kDa band in the aprotinin control did not react with the same

antibody, demonstrating the specificity of the antibody for the placental bikunin variant (102-159) (SEQ ID NO: 6).

The final preparation of placental bikunin C-terminal domain was highly pure by silver-stained SDS-PAGE (Figure 10). The overall recovery of broth-derived trypsin inhibitory activity in the final preparation was 31%. N-terminal sequencing of the purified inhibitor indicated that 40% of the protein is correctly processed to yield the correct N-terminus for placental bikunin (102-159) (SEQ ID NO: 6) while about 60 % of the material contained a portion of the yeast α-mating factor. The purified material comprised an active serine protease inhibitor exhibiting an apparent Ki of 0.35 nM for the *in vitro* inhibition of plasma kallikrein.

In conclusion, the accumulation both of a protease inhibitor activity and a protein immunochemically related to synthetic bikunin (102-159) (SEQ ID NO: 6) in fermentation broth as well as the isolation of placental bikunin (102-159) (SEQ ID NO: 6) from one of the transformed lines provided proof of expression of placental bikunin in the recombinant yeast strains described herein, showing for the first time the utility of yeasts for the production of placental bikunin fragments.

Additional constructs were prepared in an effort to augment the expression level of the Kunitz domain contained within placental bikunin 102-159 (SEQ ID NO: 6), as well as to increase the yield of protein with the correct N-terminus. We hypothesized that the N-terminal residues of placental bikunin 102-159 (SEQ ID NO: 6) (YEEY--) may have presented a cleavage site that is only poorly recognized by the yeast KEX-2 protease that enzymically removes the yeast a-factor pro-region. Therefore, we prepared yeast expression constructs for the production of placental bikunin 103-159 (N-terminus of EEY...), 101-159 (N-terminus of NYEEY...) and 98-159 (DMFNYEEY..) in order to modify the P' subsites surrounding the KEX-2 cleavage site. To attempt to augment the levels of recombinant protein expression, we also used the yeast preferred codons rather than mammalian preferred codons in preparing some of the constructs described below. The constructs were essentially prepared as described above for placental bikunin 102-159 (SEQ ID NO: 6) (defined as construct #1) but with the following modifications:

G43 Cont

Construct #2 placental bikunin 103-159, yeast codon usage
A 5' sense oligonucleotide

Page 48, line 19, to page 49, line 5:

G44 GAAGGGGTAA GCTTGGATAA AAGAGAAGAA TACTGTACTG CTAATGCTGT TACTGGTCCA TGTAGAGCTT CTTTTCCAAG ATGGTACTTT GATGTTGAAA GA (SEQ

ID NO.: 55)

and 3' antisense oligonucleotide

ACTGGATCCT CATTGGCGAA AACATCTCAA CATACAGGCT TCTTCAGATC TGTAAGAATT TTTATTACCT CTACAACCAC CGTAAATAAA ATTATTACAA GAATTTCTTT CAACATCAAA GTACCATCT (SEQ ID NO.: 56)

G44

were manipulated as described for the production of an expression construct (construct #1 above) for the expression of placental bikunin 102-159 (SEQ ID NO: 6)

Construct #3 placental bikunin 101-159, yeast codon usage

A 5' sense oligonucleotide

GAAGGGGTAA GCTTGGATAA AAGAAATTAC GAAGAATACT GTACTGCTAA TGCTGTTACT GGTCCATGTA GAGCTTCTTT TCCAAGATGG TACTTTGATG TTGAAAGA (SEQ ID NO.: 57)

and the same 3' antisense oligonucleotide as used for construct #2, were manipulated as described for the production of an expression construct (construct #1 above) for the expression of placental bikunin 102-159 (SEQ ID NO: 6).

Page 49, lines 18-26:

Yeast strain SC101 (MATα, ura 3-52, suc 2) was transformed with the plasmids containing each of the above cDNAs, and proteins were expressed using the methods that were described above for the production of placental bikunin 102-159 (SEQ ID NO: 6) with human codon usage. Approximately 250 ml of each yeast culture was harvested, and the supernatant from centrifugation (15 min x 3000 RPM) separately subjected to purification over 1 ml columns of kallikrein-sepharose as described above. The relative amount of trypsin inhibitory activity in the applysate, the amount of purified protein recovered and the N-terminal sequence of the purified protein were determined and are listed below in Table 7.

G45

Page 55, line 28, to page 56, line 3:

As expected based on the N-terminal sequencing results described above, the purified protein reacted with an antibody elicited to placental bikunin (7-64) (SEQ ID NO: 4) to yield a band with the same Mr (Figure 12A) as observed for the purified preparation detected on gels by silver stain (Figure 7). However, when the same preparation was reacted with an antibody elicited to synthetic placental bikunin (102-159) (SEQ ID NO: 6), a band corresponding to the full length protein was not observed. Rather, a fragment that co-migrated with synthetic bikunin (102-159) (SEQ ID NO: 6) of approximately 6 kDa was observed. The simplest interpretation of these results is that the purified preparation had undergone degradation subsequent to purification to yield an N-terminal fragment comprising the N-terminal domain and a C-terminal fragment comprising the C-terminal domain. Assuming that the fragment reactive against antiserum to placental bikunin (7-64) (SEQ ID NO: 4) is devoid of the C-terminal end of the full length protein, the size (24 kDa) would suggest a high state of glycosylation.

G46

Page 56, lines 4-6:

Table 6. below shows the potency of *in vitro* inhibition of various serine proteases by placental bikunin. Data are compared with that obtained with aprotinin (~~Trasylol®~~ TRASYLOL®).

G47

Page 56, line 17-27:

A multiple tissue northern was purchased from Clontech which contained 2 μg of polyA+ RNA from human heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas. Two different cDNA probes were used: 1) a gel purified cDNA encoding placental bikunin (102-159) (SEQ ID NO: 6); 2) the 780 base pair PCR-derived cDNA (Figure 4E) liberated from a TA clone by digestion with EcoRI and gel purified. Each probe was labeled using ^{32}P-dCTP and a random priming labeling kit from Boehringer Mannheim Biochemicals (Indiana), then used to hybridize to the multiple tissue northern according to the manufacturers specifications. Autoradiographs were generated using Biomax film with an 18 hr exposure time, and developed using a Umax Scanner and scanned using Adobe Photoshop.

G48

Page 56, line 29, to page 57, line 4:

Results. The pattern of tissue expression observed using a placental bikunin (102-159) (SEQ ID NO: 6) probe (Figure 11A) or a larger probe containing both Kunitz domains of placental bikunin (Figure 11B) was essentially the same as might be expected. The placental bikunin mRNA was most abundant in pancreas and placenta. Significant levels were also observed in lung, brain and kidney, while lower levels were observed in heart and liver, and the mRNA was undetectable in skeletal muscle. The transcript size was 1.95 kilobases in all cases, in close agreement with the predicted size of placental bikunin deduced both from EST overlay and cloning of full length cDNA described in preceding sections.

G49

Page 57, line 37 to page 38, line 11:

Purification and properties of Placental Bikunin (1-170) (SEQ ID NO: 150) highly purified from a Baculovirus / Sf9 expression system

A large fragment of Placental bikunin containing both Kunitz domains (Placental Bikunin 1-170 (SEQ ID NO: 150)) was expressed in Sf9 cells as follows. Placental bikunin cDNA obtained by PCR (Figure 4E) and contained within a TA vector (see previous Examples) was liberated by digestion with HindIII and Xba1 yielding a fragment flanked by a 5' XbaI site and 3' HindIII site. This fragment was gel purified and then cloned into the M13mp19 vector (New England Biolabs, Beverly, MA). In vitro mutagenesis (Kunkel T.A., (1985) Proc. Natl. Acad. Sci. USA, 82: 488-492) was used to generate a Pst1 site 3' to the XbaI site at the 5' end, but 5' to the sequence encoding the ATG start site, natural placental bikunin signal peptide and mature placental bikunin coding sequence. The oligonucleotide used for the mutagenesis had the sequence:

G50

Page 58, lines 22-28:

The stop codon was in frame with the sequence encoding placental bikunin and caused termination immediately following the Lysine at amino acid residue 170, thus encoding a truncated placental bikunin fragment devoid of the putative transmembrane domain. The product from digestion with Pst1 and BglII was isolated and cloned into the BacPac8 vector for expression of Placental bikunin fragment (1-170) (SEQ ID NO: 150) which contains both

G51

G51 Kunitz domains but which is truncated immediately N-terminal to the putative transmembrane segment.

Page 59, lines 13-23:

Chromatography of the crude material over an immobilized bovine pancreatic kallikrein affinity column selectively isolated 0.013 % of the protein and 0.67 % of the trypsin inhibitory activity present. The majority of the trypsin inhibitory activity present in the starting supernatant did not bind to the immobilized kallikrein and is not related to bikunin (results not shown). G52 Subsequent chromatography using C18 reverse-phase yielded a further purification of 5-fold, with a recovery of 0.2%. The final preparation was highly pure by SDS-PAGE (Figure 13), exhibiting an Mr of 21.3 kDa, and reacted on immunoblots to rabbit anti-placental bikunin 102-159 (SEQ ID NO: 6) (not shown). N-terminal sequencing (26 cycles) yielded the expected sequence for mature placental bikunin (Figure 4F) starting at residue +1(ADRER....) , showing that the signal peptide was correctly processed in Sf9 cells.

Page 60, lines 3-5:

Thus N-terminii corresponding to each of the expected four fragments were recovered. G53 This confirms that the Sf9 expressed protein contained the entire ectodomain sequence of placental bikunin (1-170) (SEQ ID NO: 150).

Page 61, lines 1-2:

G54 **Table 9 Comparisons of Ki values for the inhibition of various proteases by recombinant placental bikunin (1-170) (SEQ ID NO: 150) or aprotinin**

Page 61, lines 6-13:

The results show that recombinant bikunin can be expressed in insect cells to yield an G55 active protease inhibitor that is effective against at least five different serine protease inhibitors. Recombinant bikunin was more potent than aprotinin against human plasma kallikrein, trypsin and plasmin. Surprisingly, the recombinant bikunin was more potent that the synthetically derived bikunin fragments (7-64) (SEQ ID NO: 4) and (102-159) (SEQ ID NO: 6) against all

G55 enzymes tested. These data show that recombinant bikunin is more effective than aprotinin, using *in vitro* assays, and that one would expect better *in vivo* potency.

Page 61, line 14 tp page 62, line 4:

Besides measuring the potencies against specific proteases, the capacity of placental bikunin (1-170) (SEQ ID NO: 150) to prolong the activated partial thromboplastin time (APTT) was evaluated and compared with the activity associated with aprotinin. Inhibitor was diluted in 20 mM Tris buffer pH 7.2 containing 150 mM NaCl and 0.02% sodium azide and added (0.1 ml) to a cuvette contained within an MLA Electra^R 800 Automatic Coagulation Timer coagulometer (Medical Laboratory Automation, Inc., Pleasantville, N.Y.). The instrument was set to APTT mode with a 300 sec. activation time and the duplicate mode. Following addition of 0.1 ml of plasma (Specialty Assayed Reference Plasma lot 1-6-5185, Helena Laboratories, Beaumont, TX), the APTT reagent (Automated APTT-lot 102345, from Organon Teknika Corp., Durhan, NC) and 25 mM CaCl2 were automatically dispensed to initiate clotting, and the clotting time was monitored automatically. The results (Figure 14) showed that a doubling of the clotting time required approximately 2 μM final aprotinin, but only 0.3 μM Sf9 derived placental bikunin. These data show that placental bikunin is an effective anticoagulant, and usefull as a medicament for diseases involving pathologic activation of the intrinsic pathway of coagulation.

G56

Page 62, lines 17-26:

Aqueous formulations of Bikunin (5 and 50 ug/mL (SEQ ID NO: 52)) and amiloride (obtained from Sigma Chemicals, St. Louis, MO, USA)(100 uM) were prepared, sterile filtered and endotoxin tested prior to use. These formulations were prepared in Hank's Balanced salt solution (HBSS) and contained 137 mM NaCl, 3 mM KCl, 3 mM KH_2PO_4, 8 mM Na_2HPO_4, 0.2% Tween-80, pH 7.1) was prepared, sterile filtered and endotoxin tested for use in this example. HBSS was used as a control solution. ~~Hypnorm®~~ HYPNORM® (Fentanyl citrate 0.315 mg/mL and Fluanisone 10 mg/mL) was obtained from Janssen Animal Health and ~~Hypnovel®~~ HYPNOVEL® (Midazolam 5 mg/mL) was obtained from Roche. Male Dunkin-Hartley guinea pigs (550-750 g) were supplied by David Hall, UK. Thermistor probes were obtained from Kane-May Ltd, UK.

G57

Page 62, lines 29-34:

G58

Animals were anaesthetised using halothane. Once a satisfactory level of anaesthesia was induced a small incision was made below the lower jaw. The trachea was exposed and 100 ul volume of vehicle, bikunin (0.5 ug or 5 ug) or amiloride (100 uM) was instilled onto the tracheal surface using a needle and syringe. Once injected, the skin incision was sealed using ~~Vetbond®~~ VETBOND® (cyanocacrylate tissue glue). The animals were then allowed to recover.

Page 62, line 37 to page 63, line 12:

G59

Two hours following agent treatment, guinea-pigs were anaesthetised for a second time with ~~Hypnom®~~ HYPNORM® and ~~Hypnovel®~~ HYPNOVEL® and immobilised in a supine position. Rectal temperature, measured with a thermistor probe was maintained at 37° C by manual adjustment of a heat lamp. A ventral midline incision was made from the lower jaw to the clavicles. Using blunt dissection a length of trachea was exposed and bisected at the upper edge of the sternum. The external jugular vein was exposed and cannulated. The caudal part of the trachea was then cannulated to allow the animal to spontaneously breath room air. The animal was then placed supine and its body temperature maintained using the heat lamp. 20 min. following induction of i.v. anaesthesia the tracheal agar electrode was inserted into the cephalad trachea and tracheal potential difference was measured for 60 minutes. The reference electrode was placed under cephalad trachea in contact with the trachea cartilage. The wound site was covered to prevent drying.

Page 63, lines 30-37:

G60

A Bikunin formulation (50 ug/mL Bikunin (SEQ ID NO: 52) was prepared in HBBS containing 137 mM NaCl, 3 mM KCl, 3 mM KH_2PO_4, 8 mM Na_2HPO_4, 0.2% Tween-80, pH 7.1). The formulation was sterile filtered and endotoxin tested prior to use in this example. HBSS was used as a control solution. ~~Hypnorm®~~ HYPNORM® (Fentanyl citrate 0.315 mg/mL and Fluanisone 10 mg/mL) was obtained from Janssen Animal Health and ~~Hypnovel®~~ HYPNOVEL® (Midazolam 5 mg/mL) was obtained from Roche. Male Dunkin-Hartley guinea

pigs (550-750 g) were supplied by David Hall, UK. Thermistor probes were obtained from Kane-May Ltd, UK.

Page 64, lines 2-7:

G61

Animals were anaesthetized using halothane. Once a satisfactory level of anaesthesia was induced, a small incision was made below the lower jaw. The trachea was exposed and 100 ul volume of vehicle or bikunin (5 ug) was instilled onto the tracheal surface using a needle and syringe. Once instilled, the skin incision was sealed using ~~Vetbond®~~ VETBOND® (cyanocacrylate tissue glue). The animals were then allowed to recover.

Page 64, lines 17-23:

G62

70 minutes following instillation of bikunin, each animal was anaesthetized for a second time using ~~Hyponorm®~~ HYPNORM® and ~~Hyponovel®~~ HYPNOVEL® and immobilized in a supine position. The first TMV measurement was made 20 minutes afterwards. Subsequent measurements were taken every 15 minutes. The procedure for TMV measurements is described, in detail, in Newton et al., "Cilia. Mucus and Mucociliary Interactions." Ed. Baum, G.L., Preil, Z., Roth, Y., Liron., Ostfield, E., Marcel Dekker. New York, 1990 and Newton et al. in *Pediatric Pulmonology* S17, Abs 364, 1998.

Page 65, lines 24-27:

G63

~~Hypnorm®~~ HYPNORM® (Fentanyl citrate 0.315 mg/mL and Fluanisone 10 mg/mL) was obtained from Janssen Animal Health and ~~Hypnovel®~~ HYPNOVEL® (Midazolam 5 mg/mL) was obtained from Roche. Male Dunkin-Hartley guinea pigs (550-750 g) were supplied by Harlan UK Ltd. Thermistor probes were obtained from Kane-May Ltd, UK.

Page 65, lines 30-34:

G64

Animals were anaesthetized using ~~Hypnorm®~~ HYPNORM® and ~~Hypnovel®~~ HYPNOVEL®. TMV was monitored using a lead collimated miniature Beta particle detector probe arranged to detect the radioactivity emitted from an injected aliquot of ^{32}P-labelled

G64 *Saccharomyces cerevisiae* as it was transported on the tracheal mucociliary layer of an anaesthetized guinea pig (Newton and Hall 1998)

Page 66, lines 24-28:

An amiloride formulation (10 mM) in water was prepared for this example. ~~Hypnorm®~~ HYPNORM® (Fentanyl citrate 0.315 mg/mL and Fluanisone 10 mg/mL) was obtained from Janssen Animal Health and ~~Hypnovel®~~ HYPNOVEL® (Midazolam 5 mg/mL) was obtained from Roche. Male Dunkin-Hartley guinea pigs (550-750 g) were supplied by Harlan UK Ltd. Thermistor probes were obtained from Kane-May Ltd, UK.

G65

Page 66, line 31 to page 67, line 2:

Animals were anaesthetized using ~~Hypnorm®~~ HYPNORM® and ~~Hypnovel®~~ HYPNOVEL®. TMV was monitored using a lead collimated miniature Beta particle detector probe arranged to detect the radioactivity emitted from an injected aliquot of ^{32}P-labelled *Saccharomyces cerevisiae* as it was transported on the tracheal mucociliary layer of an anaesthetized guinea pig. Guinea pigs were anaesthetized with ~~Hypnorm®~~ HYPNORM® and ~~Hyponovel~~ HYPNOVEL® at time 0. Amiloride (10mM x 20 min) was administered with Hyponorm® and Hypnovel at t = 0. Amiloride (10 mM x 20 min) was administered by aerosol. The first TMV measurement was made immediately afterwards and subsequent measurements were taken every 15 minutes.

G66